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Assessing the Efficacy and Biological Benefits of Withanolide-rich *Withania somnifera* Root Extract

Devaraj Reddy KN [a*], Srilakshmi Aluri [a*], Prathvi Shetty [a], Shreya Udaya [a], Shankara Prasad [a], Sudhanva MS [b] and Shobith Rangappa [b]

[a] Prakruti Products Pvt. Ltd., No. 405, Vasanthanarasapura, Phase 2, Industrial Area, Tumakuru, Karnataka, India.
[b] Adichunchanagiri Institute of Molecular Medicine, Adichunchanagiri University, B. G Nagar, Mandya, Karnataka, India.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The medicinal plant *Withania somnifera*, usually referred to as Ashwagandha, is a member of the Solanaceae family. The presence of Withanolides in the roots is responsible for a number of pharmacological effects in Ashwagandha. Withanolides have been demonstrated to be an effective neuronal, immune, anti-stress, and anti-cancer agent. However, Withanolides demonstrated limited permeability, lowering the bioavailability and efficacy of active compounds. The goal of the study was to ascertain the biological efficacy of Ashwagandha Composition, a blend of *W. somnifera* milk extract and water extract (1:1) with a high concentration of Withanolides (3-5%), at lower dosages with improved bioabsorption compared to pure Ashwagandha Hydro ethanolic extracts fortified with 2.5% withanolides. The *W. somnifera* Composition was assessed for its bioabsorption and bioefficacy by exploring its intestinal absorption capacity, Acetylcholinesterase Inhibition (82%),

**Corresponding author: E-mail: devarajreddy@prakrutees.com; rndphytotmk@prakrutees.com;*

antioxidant potential (91%), glutathione reduction potential (15.6%), anti-inflammatory activity (87.2%), and anti-cancer activity. Additionally, Ashwagandha Composition was also evaluated for its safety profile. We found *W. somnifera* Composition (1:1) is more bioavailable and showed higher biological activity than the Ashwagandha Hydro ethanolic extract fortified with 2.5% withanolides (Ashwagandha extract). Hence, the *W. somnifera* a Composition can be used as a therapeutic medication once its safety concerns are addressed by *in vivo* trials.

Keywords: Withania somnifera; withanolides; bioabsorption; bioactivity.

1. INTRODUCTION

Withania somnifera, popularly recognized as "Ashwagandha," "Indian Winter Cherry," or "Indian Ginseng," is a key therapeutic plant in Ayurveda, India's holistic medical system [1]. For millennia, it has been utilized as a Rasayana for its wide spectrum of health virtues. This herbal concoction fosters physical and mental health and elevates oneself. Tonics of this type are administered to little children, and they are also used to promote life expectancy in the middle-aged and elderly [2]. Ashwagandha is the most important Rasayana herb used in Ayurvedic therapy. It is known as "Sattvic Kapha Rasayana" herb. A significant proportion of Rasayana herbs act as anxiety relievers [3]. It is typically marketed as a churna, a finely ground powder that can be combined with ghee, water, or honey for administration. It is well-recognized for enhancing energy and stamina and for having sedative, diuretic, and anti-inflammatory properties. It promotes memory and improves neurological function [4,5]. By enhancing the reproductive system's functionality, it promotes the harmony of sexual and reproductive activities. It boosts the body's tolerance to stress due to its high adaptogenic characteristics [6]. *W. somnifera* strengthens the body's resistance to illness through increasing cell-mediated immunity. Additionally, it has strong antioxidant qualities that aid in preventing cellular damage brought on by oxidants [2]. *W. somnifera* has a long history of being used as a powerful rejuvenator, tonic, and therapy for an array of diseases. It also functions as an adaptogen and has potent anti-stress and immunostimulatory properties [4]. As a result, in the Indian Herbal System, *W. somnifera* is recognized as one of the most pivotal herbs and an ideal adaptogen.

W. somnifera has a several pharmacological effect, which are mostly ascribed to the bioactive constituents, such as Withanolides, found in the roots of the herb. The primary Withanolides isolated from the roots of *W. somnifera* are Withanoside V (WS V), Withaferin A, Withanolide A (WN A), and Withanolide B (WN B), which aggregate to about 35 Withanolides in total [5-7]. Withaferin A exhibits anti-inflammatory, anti-cancer, anti-angiogenesis, and anti-apoptotic properties. Withanolide are useful in the treatment of an array of malignancies, such as breast cancer, pancreatic cancer, and skin cancer [8]. Withanolide A is an effective neuronal, immune, and anti-stress drug. Withanolide IV and V are crucial in neuroregeneration. However, the permeability of the glycosylated and polar Withanolide glycosides WS IV and WS V is poor. As a result, the bioavailability and efficacy of active substances containing anolide glycosides are reduced [9]. Therefore, the present invention relates to making *W. somnifera* more bioavailable to the body and thereby enhancing its bioactivity.

The current study investigates the bioactivity of *W. somnifera* Composition, which is a combination of *W. somnifera* milk extract and water extract with a high concentration of Withanolides. The bioactivity of Ashwagandha Hydro ethanolic extract fortified with 2.5% withanolides (*W. somnifera* extract) was used to compare the effectiveness of this *W. somnifera* Composition. The goal of this study is to look into its effectiveness as a potent acetylcholinesterase (AChE) inhibitor, anti-oxidant, cyclooxygenase inhibitor, and anti-cancer agent.

2. METHODS

2.1 Sample Collection and Authentication

W. somnifera raw materials supplied by Star marketing, Urban Bengaluru, Karnataka, India. I was authenticated by Dr. Syed Noorie, Senior Botanist, Foundation for Revitalisation of Local Health Traditions, Bengaluru, Karnataka, India

2.2 Sample Preparation

An herbal composition was formulated with a mixture of *W. somnifera* milk extract and water

extract (1:1) with a high concentration of Withanolides by using the process discussed by Reddy et al [9]. This composition was assessed for its biological activities and efficacy was compared with the bioactivity of ashwagandha Hydro ethanolic extract fortified with 2.5% withanolides.

2.3 Predicted Bioavailability Assessment of Withanolides in *W. somnifera* Extracts Using Caco-2 Model

Cytotoxicity tests determined the non-toxic concentration for absorption trials. In three separate experiments, the permeability test was carried out in the apical-basolateral (A-B) and basolateral-to-apical (B-A) directions at 37 °C for 2 hours in a shaking incubator with 100 rpm using *W. somnifera* extracts dissolved in Hank's Balanced Salt Solution (HBSS) buffer. Reverse phase chromatography was used to analyze the transported materials using a C18 column (250mmX 4.6mm) and UV-VIS detector to quantify them (Waters HPLC system, Milford, MA, USA (mobile phase A: Anhydrous potassium diohydrogen orthophosphate; mobile phase 2: Acetonitroile). Drug absorption studies on Caco-2 cells involved Ashwagandha Composition administered at 1 mg/mL, analyzed over different time intervals. The apparent permeability coefficient of the compounds was computed [10]. The apparent permeability coefficient (Papp) was computed using MS Excel using the standard formula [11,12].

2.4 Acetylcholinesterase Inhibition Assay

The Acetylcholinesterase (AChE) Assay Kit, employing DTNB, measured AChE activity [13]. Thiolcholine levels indicated AChE inhibition, following the manufacturer's protocol (Thermo Fisher, Scientific, India). 1 µg/mL of control and sample were used for the study.

2.5 Radical Scavenging Activity

Withania somnifera Composition and extract were evaluated for antioxidant potential. 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was performed with 1 µg/mL concentration of standard. *W. somnifera* extract and composition were evaluated at different concentrations as 1, 2.5, 5 and 10 µg/µl and absorbance measurement at 517 nm to determine percentage inhibition. All chemicals and standards were purchased from Thermo Fisher, Scientific, India [14].

2.6 Glutathione Reduction Assay

Assessment of *W. somnifera* Composition's ability to generate free radicals at 1 µg/mL concentrations was measured using Glutathione Reduction Assay kit (Thermofisher, Scientifics, India). Glutathione and Ascorbic acid (Positive control) was purchased from Sr. Lifesciences, Bengaluru, India. Absorbance was measured at 412 nm for radical formation after incubation [15].

2.7 *In-vitro* Anti-inflammatory Activity

Cyclooxygenase-2 (COX-2) inhibitor screening kit (Thermofisher, Scientifics, India) measured fluorescence during prostaglandin G2 production by COX-2 enzyme. prostaglandin G2 produced was assessed for the anti-inflammatory activity of *W. somnifera* extract and composition [16].

2.8 *In-vitro* Anti-cancer Activity

3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) method was used to evaluate Ashwagandha compositions against pancreatic, breast, and colorectal cancer cells [17]. MTT *in vitro* cell proliferation assays were performed on MIA PaCa-2, MBA-MB-231, MDA-MB-468, and HCT116 cell lines. All cell lines were purchased from cell line repository, National Centre for Cell Science, Pune, India. A Tecan Microplate reader was employed to measure the absorbance at 570 nm. Megalan software (Tecan Instruments, Inc.) was used to collect the data, which was then exported in Microsoft Excel format for additional analysis [18].

2.9 Toxicity Studies

In-vitro cytotoxicity of *W. somnifera* extract and Composition against Kidney epithelial cells (Hek293T) and Skin Fibroblast cells (HFF-1) was assessed using the MTT method. These cell lines were purchased from American Type Culture Collection. Absorbance at 570 nm measured cytotoxic effects at various concentrations [17,18].

2.10 Statistical Analysis

The data were calculated and graph was prepared using MS Excel (2019) software. The mean values, standard deviation, and level of significance were calculated.

3. RESULTS AND DISCUSSION

3.1 Determining the Bioavailability of Withanolides of Ashwagandha Composition Using an *In-vitro* Absorption Model System

Withania somnifera has a multitude of pharmacological properties that are mostly due to the bioactive constituents, such as Withanolides, found in the roots of the herb. The effectiveness of bioactive Withanolide components is reliant on their absorption and transit across the intestinal epithelium. The *in-vitro* cell culture method has been touted as a potential model system for efficiently determining a drug's bioavailability. We described the bioavailability profiles of primarily Withanolides from *W. somnifera* in this study using the Caco-2 cell culture method. An important requirement for a medicine to be taken orally is that it has adequate intestinal absorption in humans; in the early phases of drug discovery, this requirement is often tested using *in vitro* techniques. There are several *in-vitro* intestinal absorption models that fall under the categories of physicochemical and biological techniques [19].

Table 1 and Fig. 1 show the results of the analysis of *W. somnifera* extract and Ashwagandha Composition's intestinal absorption in Caco-2 cells. When compared to pure Ashwagandha extracts, the Caco-2 *in-vitro* absorption study demonstrated that the synergistic combination of *W. somnifera* milk extract and act with a high concentration of Withanolides improved membrane permeability and absorption. The *W. somnifera* a component was completely absorbed by the Caco-2 cells from the medium after 5 minutes, as validated by HPLC qualitative evaluation.

At a concentration of 0.125 mg/mL, the transfer of active components from the apical to the basolateral (ab) was studied. Ashwagandha Composition was found to have the highest apparent permeability coefficient (Papp) in absorptive transport at 164.5 ± 13.5 cms^{-1} compared to the secretive direction at 89.6 ± 11.4 cms^{-1} and an efflux ratio of 0.54. Whereas Ashwagandha extract was found to have an apparent permeability coefficient (Papp) in absorptive transport as 142.2 ± 16.3 cms^{-1} compared to secretive direction as 71.2 ± 7.2 cms^{-1} and with an efflux ratio of 0.50.

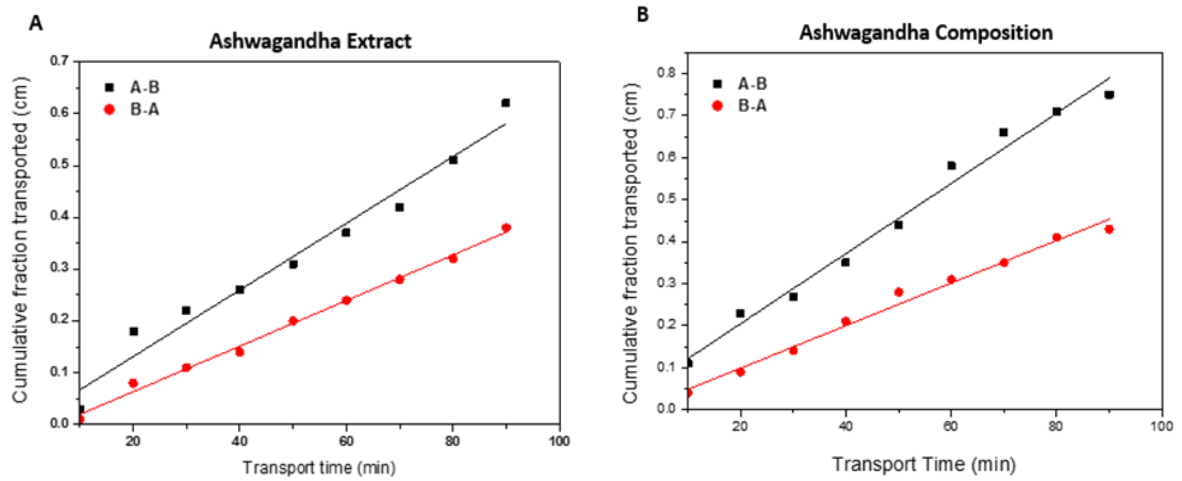

Fig. 1. Graph representing the Intestinal absorption of *W. somnifera* extract and Composition in Caco-2 cells

Table 1. Interpretation of the intestinal absorption of ashwagandha extract and ashwagandha composition in Caco-2 cells

Papp X 10^{-6} (cm/s)			
Drugs	**Concentration (mg/mL)**	**Apical to Basolateral transport**	**Basolateral to Apical transport**
***W. somnifera* extract**	0.125	142.2 ± 16.3	71.2 ± 7.2
***W. somnifera* Composition**	0.125	164.5 ± 13.5	89.6 ± 11.4

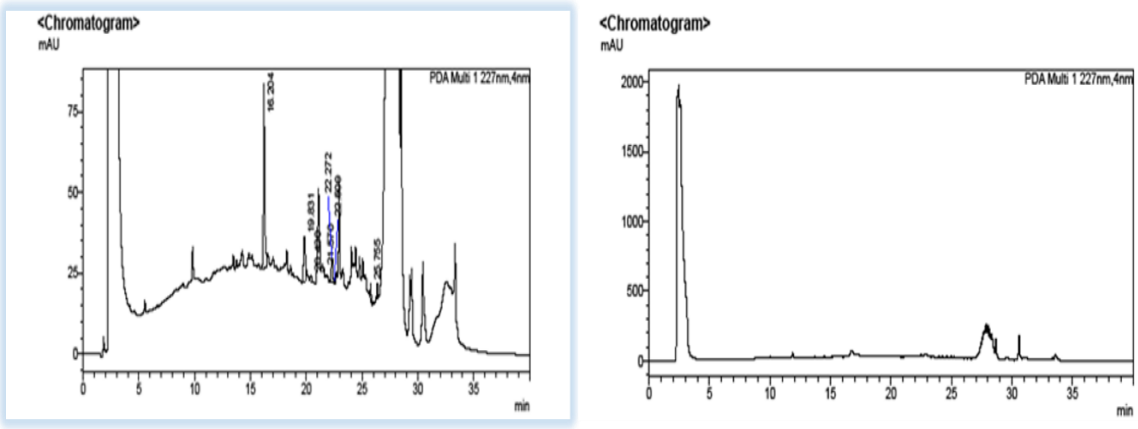

Fig. 2. ***Withania somnifera*** **composition HPLC chromatogram- qualitative determination of Withanolides in Caco-2 cells lysate and media at 5 min time interval**

At different time intervals, Caco-2 cell lysate and media samples of *W. somnifera* Composition were screened. The samples were qualitatively determined with an analytical standard by the HPLC analysis using Shimadzu's Lab Solutions software with a photodiode array detector.

The above Caco-2 *in-vitro* absorption study (Fig. 1 and Table 2) revealed that Ashwagandha Composition which is a synergistic mixture of Ashwagandha milk extract and water extract with a high concentration of Withanolides exhibited good membrane permeability and absorption compared to the as such *W. somnifera* extracts.

At the 5-minute time interval, the Ashwagandha Composition was completely absorbed by the Caco-2 cells from the media, which was confirmed by HPLC qualitative determination (Fig. 2).

3.2 Anti-acetylcholinesterase Activities

Withania somnifera is given for the treatment of memory and cognitive deficiencies caused by accidents, sickness, or simply old age. Preclinical research has suggested that ashwagandha may act as a nootropic, boosting cognitive function and memory [20]. The present study provides a novel synergistic mixture of ashwagandha milk extract and water extract with a high concentration of Withanolides, wherein the said *W. somnifera* composition proved to be a potent AChE inhibitor in comparison to *W. somnifera.* Hydro ethanolic extract and standards such as donepezil. The graph below interprets the data for the Acetylcholinesterase Inhibition Potential of *W. somnifera* Composition in comparison to *W. somnifera* extract (Fig. 3). Ashwagandha extract inhibited Acetylcholinesterase with an IC_{50} of 4.4 mg/mL and a 62% inhibition. Whereas *W. somnifera* composition was discovered to possibly inhibit Acetylcholinesterase with an IC_{50} value of 3.57 mg/mL and 84% inhibition.

3.3 Anti-oxidant Potential of the *W. somnifera* Composition

For ages, Ayurvedic medicine has employed ashwagandha to increase longevity and vitality. The roots of this plant have strong antioxidant capacity [14,21]. Through this study we assessed the antioxidant and free radical scavenging activity of *W. somnifera* Composition. A DPPH assay was performed to screen for anti-oxidant activity of the *W. somnifera* Composition which is a mixture of *W. somnifera* milk extract and water extract with a high concentration of Withanolides in comparison with *W. somnifera* Hydro ethanolic extract. The graph (Fig. 4) and table (Table 2) below provides an interpretation of the findings on the anti-oxidant potential of the *W. somnifera* Composition in comparison to the commercially available *W. somnifera* extract and other standards such as ascorbic acid. The IC_{50} value of Ashwagandha extract was found to be ~ 47.21 µg/mL. Unlike Ascorbic acid (Positive control), which had an IC_{50} value of ~7.12 g/mL, Ashwagandha Composition demonstrated potential anti-oxidant activity with an IC_{50} value of ~32.16 g/mL. The findings from this study clearly indicate that *W. somnifera* Composition has strong antioxidant properties.

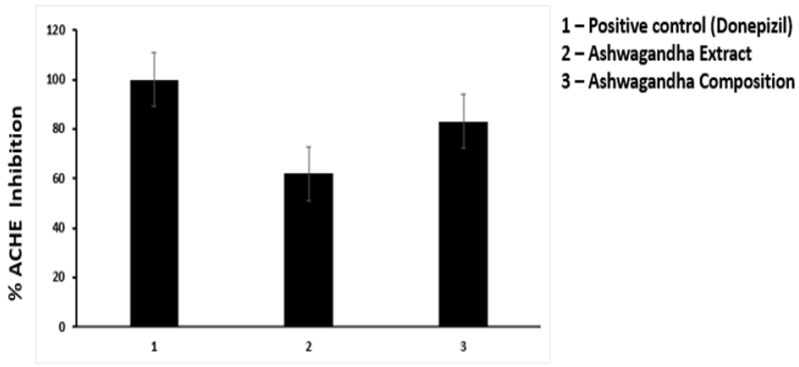

Fig. 3. Graph representing the percentage of acetylcholinesterase inhibition for *W. somnifera* extract and Composition

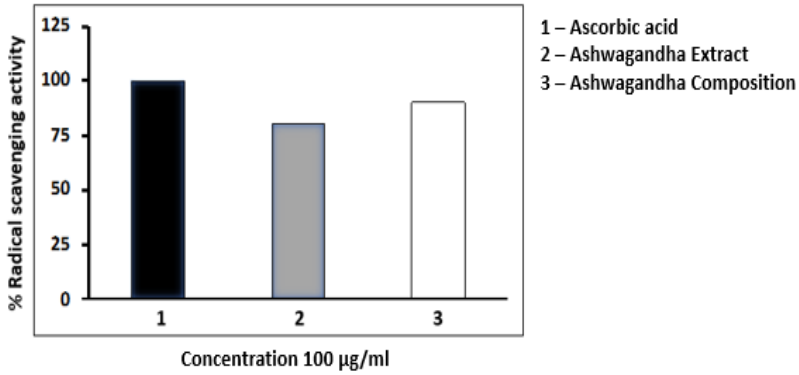

Fig. 4. Graph representing the percentage of Radical scavenging activity for *W. somnifera* extract and Composition

Table 2. DPPH free radical scavenging assay

S. No	Name of the compound	DPPH IC_{50} µg/mL
1	Ascorbic acid	~ 7.12
2	*W. somnifera* extract	~ 47.21
3	*W. somnifera* Composition	~ 32.16

3.4 Glutathione Reduction Assay of the Ashwagandha Herbal Composition

Withania somnifera is also known for its capacity to repair oxidative damage [22]. A glutathione reduction assay was performed to screen for the ability of *W. somnifera*'s Composition and extract to generate ROS. The results for the Glutathione reduction potential of *W. somnifera*'s herbal Composition in comparison to the *W. somnifera*'s extract are interpreted in the following graph (Fig. 5). Ashwagandha extract was found to reduce glutathione with ~ 11.06% inhibition at 1 µg/mL. In comparison to ascorbic acid (the positive control), which showed only 7% glutathione reduction at 1 g/mL, *W. somnifera*'s Composition was found to reduce glutathione with 15.6% inhibition at 1 g/mL.

3.5 In-vitro Anti-inflammatory Activity of the *W. somnifera* Herbal Composition

Withania somnifera, notably the roots with their distinct components, has been demonstrated to be beneficial against a variety of anti-inflammatory activity [23,24]. In the present study, An anti-inflammatory assay was performed using a COX-2 inhibitor screening kit. The anti-inflammatory activity of *W. somnifera* Composition and Aextract is represented in the graph below (Fig. 6). At 1 mg/ml, Ashwagandha

extract inhibited COX-2 with an 86.4 percent inhibition. Whereas celecoxib (positive control) exhibited only 69% COX-2 inhibition, Ashwagandha Composition was found to potentially inhibit COX-2 with 87.2 percent inhibition at 1 mg/mL.

3.6 *In-vitro* Anti-cancer Activity of the *W. somnifera* Herbal Composition

The two main active components, Withanolides and withaferins, as well as a few additional metabolites such as withanone (WN) and Withanosides, are effective against several cancer cell lines [25-27]. MTT *in vitro* cell proliferation assays were performed on MIA-PaCa-2, MBA-MB-231, MDA-MB-468, and HCT116 cell lines. *Withania somnifera* extract and Composition were checked at various concentrations, including 0.1, 0.25, 0.5, and 1 mg/mL, for their percentage inhibition of cell proliferation. A Tecan Multimode reader was used to record the data and plot a non-linear regression curve against the log concentration versus absorbance as shown in Figs. 7, 8, 9, and 10. Ashwagandha extract (Figs. 7A, 8A, 9A, and 10A) was found to effectively inhibit MIA PaCa-2, MBA-MB-231, MDA-MB-468 and HCT116 cells with an IC_{50} value of ~ 12.67, ~ 2.93, ~ 6.88, and ~ 1.47 µg/mL, respectively. Whereas, Ashwagandha Composition (Figs. 7B, 8B, 9B, and 10B) was found to effectively inhibit MIA PaCa-2, MBA-MB-231, MDA-MB-468, and HCT116 cells with an IC_{50} value of ~ 8.52, ~ 1.72, ~ 1.46, and ~ 1.68 µg/mL, respectively.

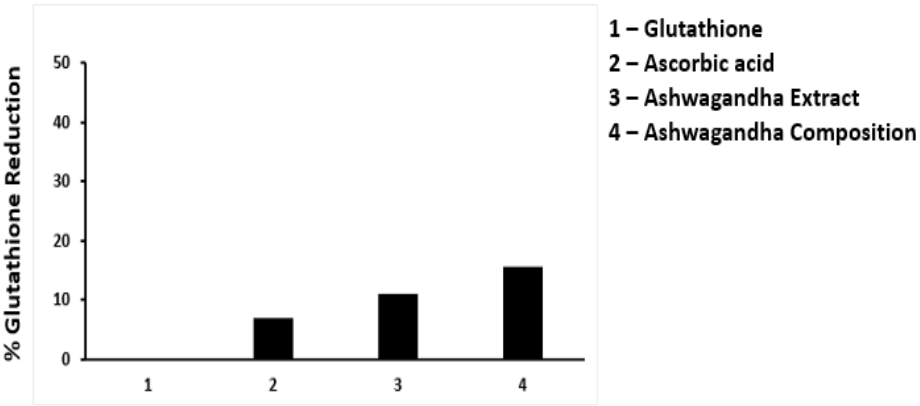

Fig. 5. Graph representing the percentage glutathione Reduction for *W. somnifera* extract and composition

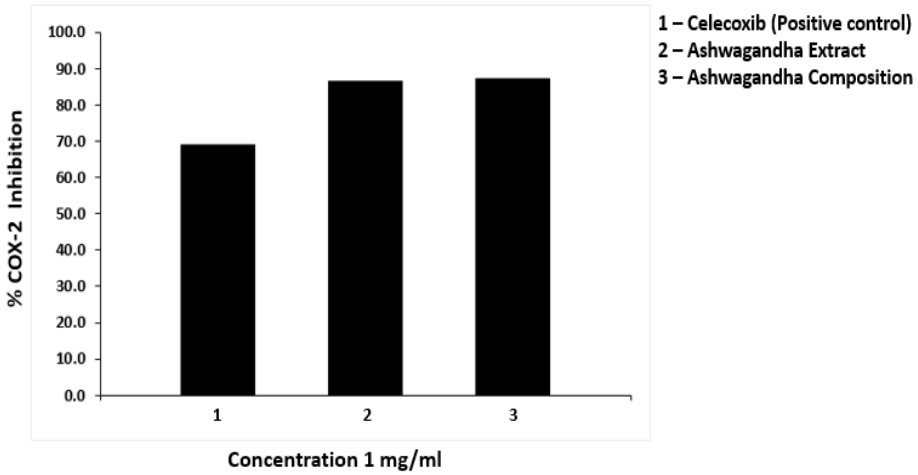

Fig. 6. Graph representing the percentage of COX-2 Inhibition for *W. somnifera* extract and Composition

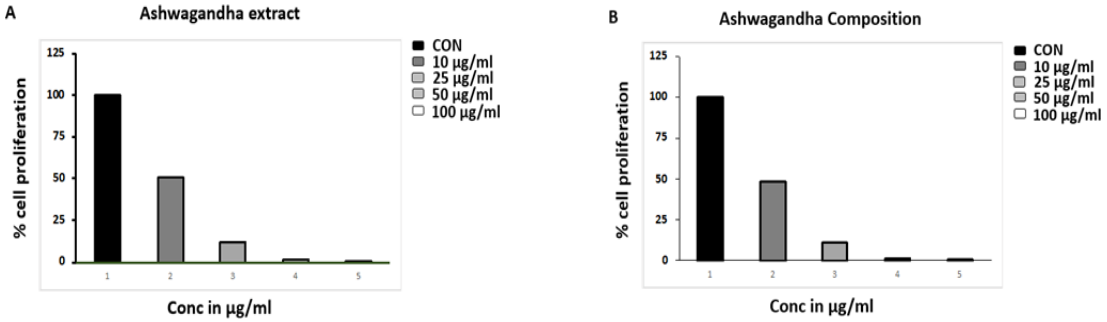

Fig. 7. Graph illustrating the percentage inhibition of proliferation of MIA PaCa-2 cells on treatment with (A) Ashwagandha extract and (B) Ashwagandha Composition

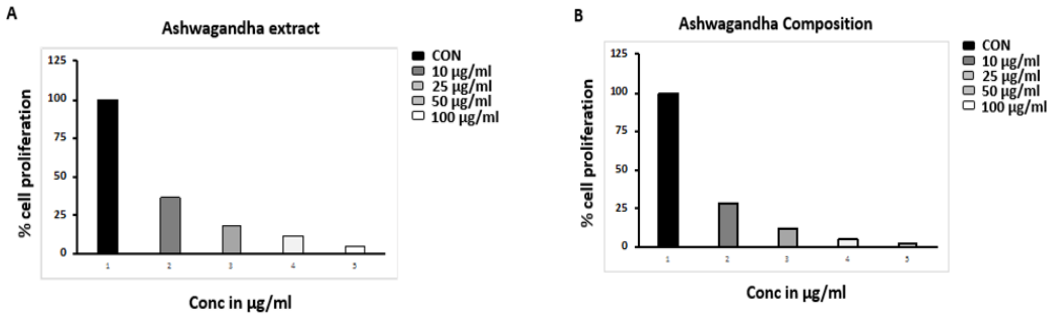

Fig. 8. Graph illustrating the percentage inhibition of proliferation of MDA-MB-231 cells on treatment with (A) *W. somnifera* extract and (B) *W. somnifera* Composition

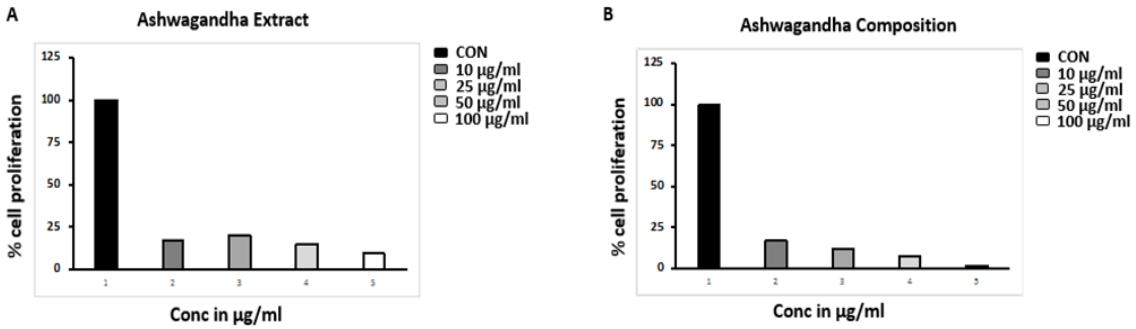

Fig. 9. Graph illustrating the percentage inhibition of proliferation of MDA-MB-468 cells on treatment with (A) *W. somnifera* extract and (B) *W. somnifera* Composition

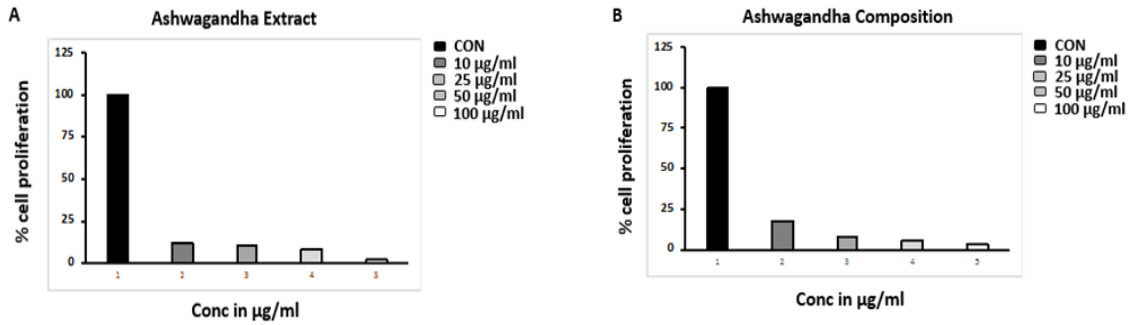

Fig. 10. Graph illustrating the percentage inhibition of proliferation of HCT116 cells on treatment with (A) *W. somnifera* extract and (B) *W. somnifera* Composition

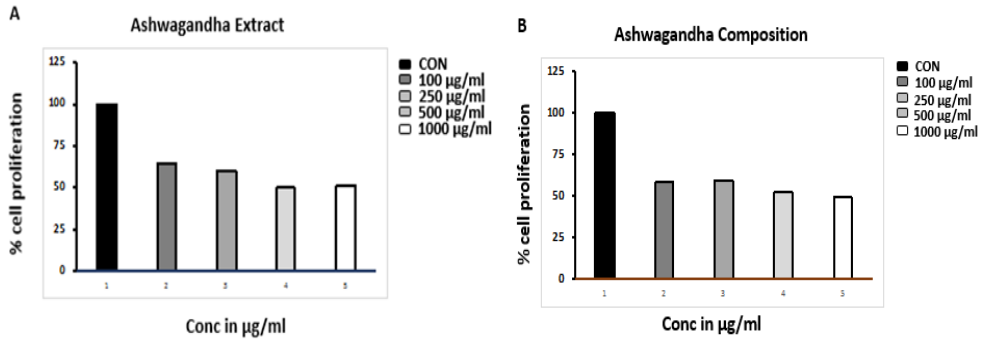

Fig. 11. Graph illustrating the percentage inhibition of proliferation of Hek293T cells on treatment with (A) *W. somnifera* extract and (B) *W. somnifera* Composition

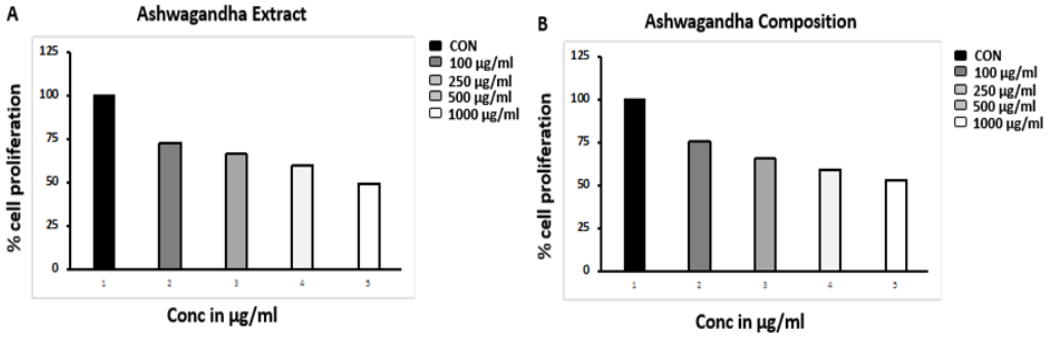

Fig. 12. Graph illustrating the percentage inhibition of proliferation of HFF-1 cells on treatment with (A) W. somnifera extract and (B) W. somnifera Composition

3.7 Toxicity Profiling of *W. somnifera* Herbal Composition

In medication research, determining a molecule's non-clinical toxicity and pharmacokinetics is crucial since it sheds light on its safety and posology in individuals. Understanding the toxicity of *W. somnifera* extract has been the focus of intensive research by several scientists around the globe [28,29]. Although WA is the part of *W. somnifera* that is most physiologically active, there is little to no evidence released regarding its safety or toxicity on oral administration. MTT *in-vitro* cytotoxicity assays were performed on Hek293T and HFF-1 cell lines. *Withania somnifera* extract and Composition were checked at various concentrations, including 0.1, 0.25, 0.5, and 1 mg/mL, for their percentage inhibition of cell proliferation. A Tecan Multimode reader was used to record the data and plot a non-linear regression curve against the log concentration versus absorbance, as shown in Figs. 11 and 12. *W. somnifera* extract (Figs. 11A and 12A) was found to be non-toxic against Hek293T and HFF-1 with an IC_{50} value of ~ >1 mg/mL and ~ 958.5 µg/mL, respectively. In contrast, *W. somnifera* Composition (Figs. 11B and 12B) was found to be safe and non-toxic against Hek293T and HFF-1, with IC_{50} values of ~> 1 mg/mL and ~1 mg/mL, respectively.

Withania somnifera is a common folk treatment in India, where people utilize it as an aphrodisiac for young people and as the finest tonic for the elderly and young. Previous research has yielded pure Withanolides with little toxicity. Although the key downside of the previous study is its low bioavailability, Due to its decreased intestinal absorption, this limits its ability to perform therapeutically [30]. The current study aims to increase the bioavailability of active ingredients in *W. somnifera* extract by combining *W. somnifera* milk water and water extract with a high concentration of Withanolides. This helps to fully harness its therapeutic potential. However, further *in vivo* studies should be explored to completely eliminate the toxicity concerns and confirm its safety for clinical use.

4. CONCLUSION

The growing incidence of cancer-related mortality necessitates the development of innovative chemopreventive medicines with minimal

detrimental reactions. Distinct active constituents extracted from different parts of the *W. somnifera* herb have been shown to exhibit potent anti-tumor and immunomodulating properties. A mixture of ashwagandha milk extract and water extract containing a high concentration of Withanolides was found to effectively inhibit the proliferation of MIA PaCa-2, MDA-MB-231, MDA-MB-468, and HCT116 cells, displaying significant anti-cancer properties. Additionally, the *W. somnifera* composition showed better bioabsorption and bioefficacy by exploring its intestinal absorption capacity as well as improved Acetylcholinesterase Inhibition, antioxidant potential, glutathione reduction potential and anti-inflammatory activity. Therefore, this extract can be employed as such or in the form of a concoction along with other therapeutic agents for *in-vivo* trials on humans.

COMPETING INTERESTS

Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

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